Method Article

A method for the transient inhibition of toxicity of secretory recombinant proteins, exemplified by bacterial alkaline phosphatase. Novel protocol for problematic DNA termini dephosphorylation

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ABSTRACT

Genes encoding proteins 'toxic' to recombinant host are difficult for cloning/expression and recombinant clones are unstable. Even tightly controlled inducible T7-lac, P_{BAD}, P_L, P_R promoters are not totally silent in an uninduced state and thus not adequate for highly toxic proteins. An innovative approach to engineering and expression of the gene, encoding bacterial alkaline phosphatase (BAP) is proposed. The native precursor enzyme contains a signal peptide at the N-terminus and is secreted to the Escherichia coli (E. coli) periplasm. The signal peptide is then removed that allows oxidation and formation of active dimers. To decrease toxicity of the bap gene, its secretion leader coding section was replaced with a N-terminal His6-tag. The gene was expressed in E. coli in a P_{BAD} vector, resulting in the accumulation of soluble His6-BAP in the cytoplasm. The His6-BAP was neutral to the cells, as no maturation was possible in the reducing cytoplasm. The purified homogenous protein was further reactivated in a redox buffer containing the protein structure stabilizing cofactors. The His6-BAP exhibited high activity. A dephosphorylation protocol for all types of DNA termini was developed. The method appears well suited for the industrial production of BAP and can be applied to other problematic proteins.

- *Efficient toxic gene expression*
- *Novel approach to toxic gene cloning, engineering, expression, purification and reactivation of the transiently inactivated enzyme*
- *Scaled-up production of ultrapure BAP*
- *Improved protocol for all types of DNA termini dephosphorylation*

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Specifications Table

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Introduction

The biotechnological production of toxic recombinant enzymes requires special measures to prevent host death before adequate expression levels are obtained. Thus the optimization of a maximum possible number of parameters such as: microbial cell cultivation conditions, induction timing, temperature, and media composition are required. As the degree of harm to a recombinant host action varies amongst toxic proteins, for those that are moderately detrimental, the above measures can be sufficient. For those proteins that are extremely toxic, even the tightly controlled pET-series expression vectors, containing T7-lac promoter are inadequate, as every expression system has a certain degree of leaky transcription/translation. An example of such an extremely toxic protein is BAP from *E. coli.* Alkaline phosphatases (ALPs) (EC 3.1.3.1) are commonly found in nature: in bacteria, shrimp, human tissues or calf intestines [1,2]. ALPs are useful molecular tools [2] and are widely used for: enzyme immunoassays, clinical diagnosis, milk industry, cosmetics, phosphatase-mediated bioremediation of toxic heavy metals, as therapeutic drugs for LPS-mediated diseases and as biofertilizer [3–6]. The major function of *E. coli* BAP is to provide inorganic phosphate when the environment is deprived of this compound [7]. Additionally, BAP is Pt-dependant, H_2-evolving hydrogenase, which catalyses the oxidation of Pt to phosphate and molecular H_2 [8]. APS are almost exclusively homodimeric metalloproteins. Their common architecture includes each catalytic site containing three metal ions: two Zn^{2+} and one Mg^{2+} [9,10]. Furthermore, to acquire a catalytically active conformation, they need disulfide bridges, among other modifications. In the active state, dimeric BAP contains a Zn^{2+} cation occupying active sites A and B, while Mg^{2+} occupies site C, thus the enzyme has the configuration (ZnAZnBMgC)2. Formation of disulfide bridges are essential for mature BAP activity [11]. BAP exhibits a wide substrate specificity, conducting: the hydrolysis of a variety of phosphoric acid monoesters, including 5′ termini of DNA, RNA, nucleotides. Moreover, it hydrolyses more 'exotic' substrates: oxyphosphate monoesters [12,13], *O*- and *S*-phosphorothioates [14–16], thiophosphates and phosphates [17,18] and phosphoramidates [13]. Such a broad specificity would make such an enzyme a perfect 'cell killer' due to the degradation of DNA, RNA, ATP and other vital phosphorylated cellular molecules. Thus Nature has 'invented' a method for producing inactive BAP, by adding an N-terminal secretion peptide and expressing such a precursor protein in the reducing, cytoplasmatic environment, thus preventing the formation of disulphide bridges. The BAP precursor needs to be transported to the periplasm, where the leader peptide can be clipped-off and the protein subjected to oxidation to form disulfide bridges. However, this natural route does not result in the high expression of the enzyme. As BAP is widely used for the removal of 5′ phosphates from linearized vectors during cloning, detecting PCR products, primer labelling and immunoassays, for biotechnology purposes the high biosynthesis and purification of ultra-pure
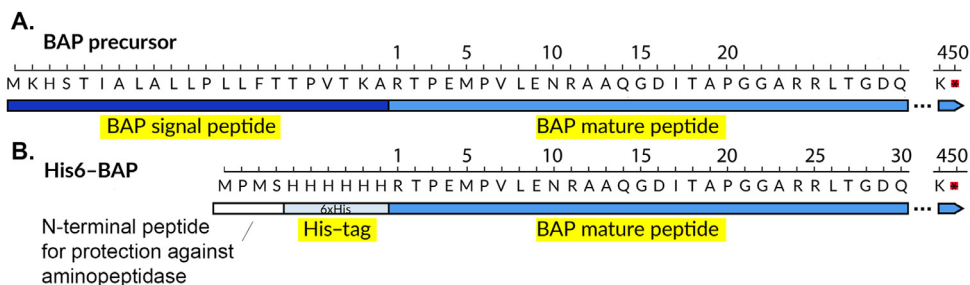
Fig. 1. The principle of gene modification for the development of the method for transient inactivation of toxic enzyme, exemplified by *bap* gene.

enzymatic product is desired. Thus we described a successful alternative strategy for the biosynthesis of BAP with transiently inhibited 'toxicity' to the recombinant *E. coli* host [19]. The strategy (Fig. 1) is based on replacing the leader-coding DNA with a His6-tag coding DNA, expression of the inactive 'mature' enzyme - leaderless His6-tagged BAP in the *E. coli* cytoplasm in the cytoplasm and *in vitro* reactivation. We anticipate that the developed method will be useful for the biotechnology production of other periplasm-secreted toxic proteins/enzymes.

Materials

Bacterial strains, plasmids, media and reagents

E. coli HB101 {F^- *mcr*B *mrr hsd*S20(r_B^- m_B^-) *rec*A13 *leu*B6 *ara*-14 *pro*A2 *lac*Y1 *gal*K2 *xyl*-5 *mtl*-1 *rps*L20(Sm^R) *gln*V44 λ^-} (Thermo Fisher Scientific (MA, USA)/GIBCO BRL) was used as a *phoA* gene source. *E. coli* DH5α {F^- Φ80Δ*lacZ*ΔM15 Δ (*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_{K-}, m_{K+}) *phoA* *sup*E44 λ-*thi*-1, *gyr*A96, *rel*A1} (Life Technologies, Gaithersburg, MD, USA) was used for transformation and DNA isolation. Bacteria were cultivated in 2xYT medium [20]. For protein expression, *E. coli* TOP10 {F^- *mcr*A Δ(*mrr*-*hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara*-*leu*)7697 *gal*U *gal*K *rps*L(Str^R) *end*A1 *nup*G λ^-} was used (Invitrogen, CA, USA). The bacteria were grown in Terrific Broth (TB) medium [20] supplemented with ampicillin at 100 μg/ml and 0.2% maltose. Difco media components were from Becton-Dickinson (Franklin Lakes, NJ). The Pwo Polymerase and DNA purification kits were from BLIRT (Gdansk, Poland). BsaI, NcoI, EcoRI, KpnI, SmaI and HindIII restriction endonucleases were from New England Biolabs (Ipswich, MA, USA). DNA standards, protein standards, the vector pBADmycHisA (Ap^R, colE1 *ori*, P_{BAD} promoter), Pierce^{TM} BCA Protein Assay Kit were from Thermo Fisher Scientific. Synthesis of PCR primers and DNA sequencing were conducted by Genomed (Warsaw, Poland). Ni Sepharose 6 Fast Flow chromatographic resin (GE Healthcare, cat. no GE17-5318-06), alkaline phosphatase from *E. coli* (cat. no P4252), glutathione oxidized form (cat. no G4626), SIGMAFAST^{TM} Protease Inhibitor Tablets (cat. no S8820), reagents for Glycine with Zinc Enzymatic Assay, SIGMAFAST^{TM} p-Nitrophenyl phosphate (pNPP) tablets (cat. no N2770) and all the other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

Software

The genetic maps of the recombinant clones were prepared using SnapGene software version 5.1.5 (http://www.snapgene.com).

Cloning of engineered *his6-phoA gene*

Leaderless *phoA* gene (wt phoA gene GenBank M29663.1 protein id AAA24363.1) was PCR amplified from the *E. coli* genome through the use of a mutagenic primer introducing the DNA segment
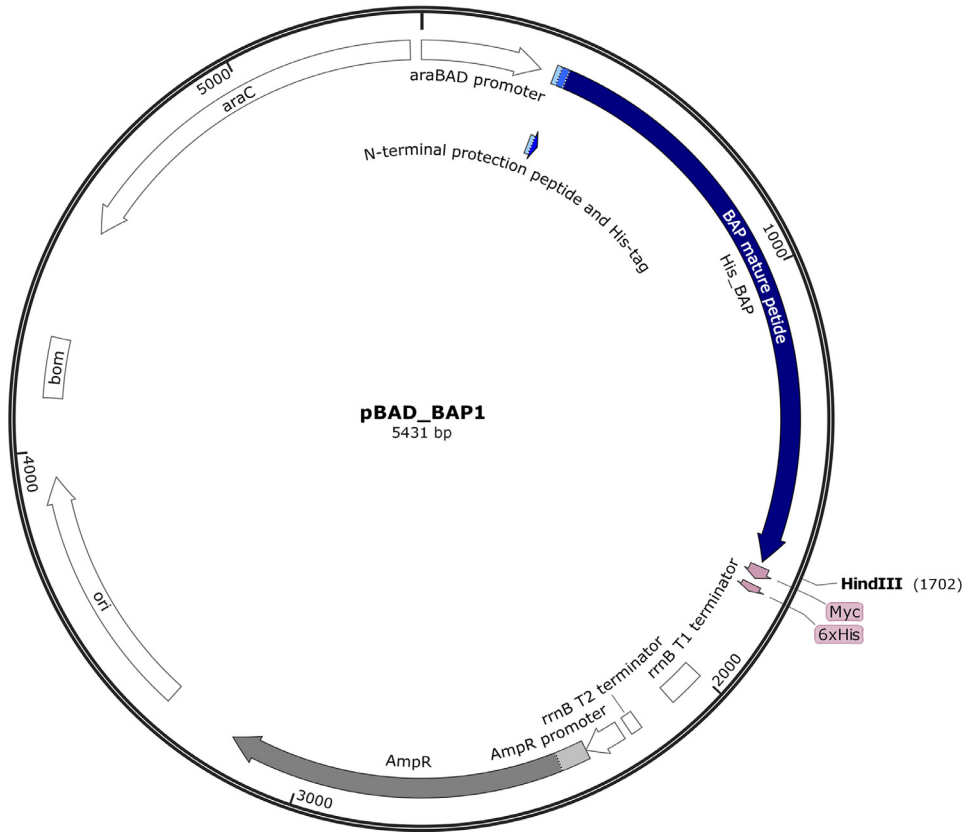
Fig. 2. Genetic map of pBAD_BAP1 expression plasmid. The map was prepared using SnapGene software version 5.1.5 (http://www.snapgene.com).

coding for His6-tag at the N-terminus in place of the secretion leader. The PCR amplifications were performed in 50 µl samples in a thermocycler (Applied Biosystems, CA, USA) and contained: 1 × Pwo PCR Buffer, 0.2 mM of each dNTP, 2 µM of each primer, 100 ng *E. coli* HB101 genomic DNA, 1 mM MgCl$_2$ and 1 unit of Pwo DNA polymerase. The following mutagenic primers were used for PCR: 5′-CCCC<u>GGTCTC</u>TC*ATG*CCAATGTCTCACCACCATCACCACCA**TAGAACACCAGAAATGCCTGT-**3′ and 5′-CACGCCGGGC<u>AAGCTT</u>*TTA***TTTCAGCC-**3′. Restriction sites BsaI and HindIII are underlined, START and STOP codon are marked in italics, leaderless wt coding segments in bold, an aminopeptidase protection segment and his6-tag are in regular letters. The PCR cycling profile was: 94 °C for 3 min, 80 °C for 20 s (addition of Pwo DNA polymerase), 94 °C for 30 s, 51 °C for 30 s, and 72 °C for 90 s (for 30 cycles); 72 °C for 3 min. PCR products were Clean-Up kit purified and digested with BsaI and HindIII. The BsaI cleavage results in 4-nt cohesive ends, which can be ligated to the NcoI-generated restriction fragments. An arabinose-regulated expression vector pBADmycHisA [21] was cleaved with NcoI and HindIII. Both the digested PCR and the vector were subjected to 1.2% agarose gel electrophoresis, purified and ligated using T4 DNA ligase at 16 °C overnight and transformed into *E.coli* DH5α competent cells. The recombinant bacterial cells were cultured on solid 2xYT medium with 100 µg/ml of ampicillin, at 28 °C. Multiple clone selection was facilitated by PCR screening of bacterial colonies using original mutagenic primers, clone plasmid DNA was isolated and sequenced. The pBAD_BAP1 clone map is shown in Fig. 2.

Expression of the recombinant, engineered his6-phoA *gene in* E. coli

E. coli BL21(DE3) were electroporated with a positive clone of pBAD_BAP1 and mini-scale expression was performed in 50 ml TB media supplemented with 100 μg/ml of ampicillin, at 28 °C with vigorous aeration. P_{BAD} promoter induction was conducted by the addition of 0.2% arabinose, when the A_{600} reached 0.8. The culture was further cultivated for 19 h at 37 °C. Bacterial cells from the control, non-induced and induced cultures were subjected to SDS/PAGE electrophoresis. The gels were analysed for the presence of the band size of ~40 kDa and using the enzymatic p-nitrophenol assay. Selected high level expression clones were used for scaling-up the biosynthesis of His6-BAP, which included: (*i*) cultivation of *E. coli* TOP10[pBAD_BAP1] in 0.5 L media containing 100 μg/ml ampicillin in 5 L Erlenmeyer flasks at 37 °C with vigorous aeration (200 rpm) until A_{600} reached 0.5 and arabinose was added to 0.02% for the induction of the P_{BAD} promoter. The bacterial cultures were then continued for next 18 h; (*ii*) cultivation of *E. coli* TOP10[pBAD_BAP1] in 5 L TB media supplemented with ampicillin at 100 μg/ml in a New Brunswick Scientific BioFlo115 fermenter at 28 °C with vigorous aeration until A_{600} reached 1.0, then arabinose was added to 0.02% and further expression was conducted for 5 h at 37 °C.

Purification, reactivation, enzymatic activity, DNA termini dephosphorylation of the recombinant His6-BAP

The recombinant His6-BAP isolation protocol [19] employed three simple steps, which included: (*i*) removal of nucleic acids and acidic proteins with polyethyleneimine (PEI); (*ii*) ammonium sulphate precipitation and (*iii*) metal affinity chromatography on Ni Sepharose 6 Fast Flow with immobilised Ni^{2+} ions.

E coli *cells lysate preparation*

Recombinant *E. coli* TOP10[pBAD_BAP1] bacteria were centrifuged. The 2.5 g of bacterial pellet was obtained. Then 25 ml of cold lysis buffer was added [30 mM Tris–HCl pH 8.0 at 20 °C, 30 mM NaCl, 5% glycerol, 3 mM 2-mercaptoethanol (βME), protease inhibitors]. The bacterial pellet was carefully suspended in the lysis buffer and 0.5 mg/ml of hen egg lysozyme was added. The mixture was stirred for 30 min at 4 °C. Crystal NaCl was added to a final 250 mM concentration. The obtained suspension was then subjected to sonication (15 × 1 min pulses separated by 1 min breaks) at 0 °C in an ice bath. Absorbance of the samples taken after every sonication pulse was measured at 280 nm using a spectrophotometer. The sonication process was continued until no increase in A_{280} was detected. The resulting cell lysate was centrifuged (19,650 x g; 4 °C; 30 min). Average yield from several purification runs was app. 160 mg His6-BAP /1 L bacterial culture.

PEI and ammonium sulphate fractionation

To the cell lysate, 10% (v/v) PEI solution (pH 7.5) was slowly added to a final 1% (v/v) concentration and the suspension was stirred for 30 min. The precipitated complexes were removed by centrifugation (19,650 x g; 4 °C; 30 min). Solid ammonium sulphate was added to the obtained supernatant (to a final concentration of 0.5 g/ml). The suspension was incubated overnight on a magnetic stirrer at 4 °C. Ammonium sulphate precipitated proteins were collected using centrifugation (19,650 x g; 4 °C; 30 min), the supernatant has been withdrawn and the pellet was solubilized in 30 ml of the N buffer [50 mM K/PO4 pH 8.0, 20 mM imidazole, 5% glycerol, 3 mM βME, 0.02% Triton X-100, protease inhibitors]. The remains of the insoluble proteins were separated by centrifugation and removed. As an excess of ammonium sulfite was used, the His6-BAP recovery was essentially quantitative, with yields over 95%.

Immobilized metal affinity chromatography (IMAC) on Ni sepharose 6 fast flow

The PEI and ammonium sulphate fractionation steps allowed for an effective removal of most cellular contaminants. The resulting His-BAP preparation was subjected to IMAC. Ni Sepharose 6 Fast Flow resin packed to a 40 ml column and equilibrated in the N20 buffer [20 mM K/PO4 pH 8.0, 20 mM imidazole, 0.5 M NaCl, 5% glycerol, 3 mM βME, 0.02% Triton X-100; protease inhibitors] was used for this purpose. The preparation was loaded onto the equilibrated resin and the column was
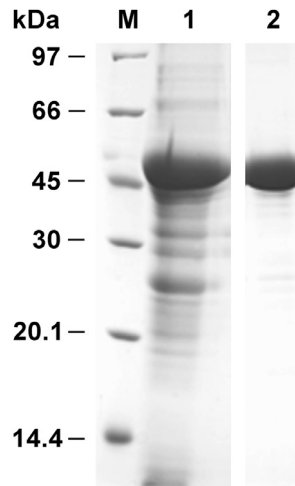
Fig. 3. Overproduction and purification of the recombinant His6-BAP. The enzyme was isolated from induced *E. coli* TOP10[*p*BAD-BAP1]. Lane M, Low Molecular Weight protein marker (GE Healthcare, cat. no 17,044,601); lane 1, crude cells extract from induced cells; lane 2, purified His6-BAP.

washed: (a) with 200 ml of the N20 buffer, (b) with 200 ml of the N40 buffer [20 mM K/PO$_4$ pH 8.0, 40 mM imidazole, 0.5 M NaCl, 5% glycerol, 3 mM βME, 0.02% Triton X-100; protease inhibitors]. The His-BAP elution was performed using 80 ml of the N500 buffer [20 mM K/PO$_4$ pH 8.0, 500 mM imidazole, 0.5 M NaCl, 5% glycerol, 3 mM βME, 0.02% Triton X-100; protease inhibitors]. The above stages provided a homogeneous His6-BAP preparation. Only in heavily overloaded sample there are visible very faint bands, apparently being a result of *in vivo* His6-BAP proteolysis, as they bind to Ni Sepharose 6 Fast Flow (Fig. 3). Similar results were obtained using the developed purification procedure for 50 g of the bacterial pellet [19]. High affinity of His6-BAP to the IMAC resin typically resulted in over 85% of the enzyme recovery.

His6-BAP enzyme activity reactivation and analysis

The purified His6-BAP was dialysed overnight at 4 °C against 2 × 1 L reactivation-oxidation buffer [20 mM K/PO$_4$ pH 7.0, 100 mM KCl, 0.2 mM MgCl$_2$, 0.2 mM ZnCl$_2$, 10% glycerol, 0.05% Tween 20, 0.05% Nonidet 40, 5 mM oxidized glutathione, 0.1 mM βME]. For the oxidation of the reduced, cytoplasmic form of His6-BAP, the oxidized form of glutathione was prepared from a fresh 0.5 M stock in water (stored at –80 °C). To the dialysed His6-BAP enzyme, oxidized glutathione was added to increase its final concentration to 20 mM and incubated overnight at 37 °C. The His6-BAP preparation was then dialyzed overnight at 4 °C against 1 L of storage buffer [100 mM KCl, 0.1 mM MgCl$_2$, 0.1 mM ZnCl$_2$, 0.05% Tween 20, 0.05% Nonidet 40, 20 mM Tris–HCl pH 7.0 at 22 °C, 5 mM K/PO$_4$, pH 7.0, 5 mM oxidized glutathione, 0.1 mM βME, 50% glycerol] and stored at –20 °C. The enzymatic activity was conducted by the use of a colorimetric assay of purified His$_6$-BAP using *p*-nitrophenyl phosphate [9,22]. A comparative assay on His6-BAP and native, commercially available alkaline phosphatase from *E. coli* of the highest quality we could locate (Sigma-Aldrich, P4252) was performed using 'Glycine with Zinc Assay' of the purified His$_6$-BAP, and was conducted as based on the rate of release of p-nitrophenol from p-nitrophenyl phosphate by following the absorbance at 405 nm [23] and essentially the same specific activities were detected. The estimated reactivation efficiency was over 90%.

His6-BAP enzyme activity toward DNA termini dephosphorylation analysis

A 'real-life' cloning assay has been conducted. To determine the His6-BAP capability to efficiently dephosphorylate DNA ends, assays were conducted in parallel with the Sigma BAP. The enzymes

were diluted to the same concentrations and used to dephosphorylate all types of DNA termini generated from the vector pUC19 linearized with: (*i*) KpnI (3′-termini); (*ii*) SmaI (blunt ends) and (*iii*) EcoRI (5′ termini). Fig. 4 shows the vector pUC19 cleavage/dephosphorylation/self-ligation assay. His6-BAP efficiently dephosphorylates the vector's DNA even at very low concentrations. In the final range tested - from 5×10^{-3} to 5×10^{-2} standard colorimetric units [23], both control Sigma BAP and His6-BAP dephosphorylated KpnI-generated 3′ sticky ends and SmaI-generated blunt ends. For cloning purposes, it is more convenient to use a DNA dephosphorylation unit, than the classical p-nitrophenyl phosphate assay. The phosphatase unit definition varies, depending on manufacturer, but in general it is based on dephosphorylation of a set amount of DNA (typically 1 μg) or pmols of 5′ends (typically 1 pmol) in a set temperature, reaction time and buffer composition. As 1 pmol of DNA ends corresponds to approx. 1 μg of a 3 kb plasmid, these definitions are close to each other. Usually they use 10–30 min reaction times and 37 °C. We have modified the unit definition used by New England Biolabs [24]. Testing a number of reaction parameters (not shown) and accounting for the time effectiveness of cloning procedures, we established a convenient practical 'cloning unit' for His6-BAP: dephosphorylation of 1 pmol of SmaI-linearized (difficult substrate) 3 kb plasmid DNA for 45 min at 55 °C, in the His6-BAP dedicated buffer. One colorimetric unit [23] corresponds to 20 DNA dephosphorylation units as determined previously (Krawczun et al. [19] in Fig. 3a, lanes 1 and 4). It is important to note that BAP thermostability allows for an increase of the reaction temperature up to approx. 80 °C [9,25], if desired. For the 3′-protruding, KpnI-linearized pUC19 DNA (Fig. 4, lanes 1–6), similar results were obtained as for SmaI (Fig. 4, lanes 7–12). For dephosphorylation of 5′ protruding EcoRI DNA termini, approx. 10 times less His6-BAP was needed (Fig. 4, lanes 13 and 14). As the enzyme is ultrapure, containing no unspecific nucleases (not shown) for maximum reduction of the cloning background, we recommend to use an excess of His6-BAP, minimum of 5–10-fold excess of His6-BAP 'SmaI cloning unit', namely over 5–10 units per 1 μg of the linearized plasmid of approx. 3 kb.

Protocol for secretory toxic gene modification to overcome toxicity

(1) Remove secretion leader-coding DNA and replace with His6-tag coding DNA by: (*i*) PCR amplification of a native gene segment coding for mature protein with the use of a mutagenic forward primer introducing an aminopeptidase protection motif MPMS and His6-tag to the N-terminus of the coded protein or (*ii*) synthesize a codon-optimized gene with the above 5′-modifications.

Note 1: *The forward primer needs to be composed of two domains: (i) the 5′-overhang: 5′-CCCC<u>GGTCTC</u>TCATGCCAATGTCTCACCACCATCACCACCAT-3′ followed by (ii) DNA segment annealing to the mature gene portion at 5′-end of the gene. The 5′ mutagenic primer portion introduces: (i) Type IIS BsaI restriction site (underlined), which further will be clipped off, leaving 5′-CATG cohesive end, compatible with commonly used for translation fusions vector's NcoI or BspHI sites; (ii) DNA coding for the MPMS segment and (iii) DNA coding for His6-tag. The reverse primer has a simple composition, it needs a DNA segment annealing to the 3′- gene terminus and a restriction site for the downstream ligation to the vector.*
Note 2: *The above protocol part assumes that minor modifications (introducing 9 amino acid residues) do not affect a given protein activity. In some cases, when the N-terminus of a protein is involved in active enzyme conformation or activity, the MPMS motif can be skipped and/or HHHHHH amino acid segment-coding DNA can by moved to the protein C-terminus coding region either via a reverse mutagenic primer or gene synthesis.*

(2) Clone the modified gene into the vector of choice.
(3) Follow the specific expression procedure for the selected vector to obtain cytoplasmatic accumulation of His6-tag equipped, leaderless protein. Usually the clipping off of leaders during translocation to the periplasm or outside a cell is required to activate the protein. Thus, it is expected that the modified toxic proteins locked in the cytoplasm will remain inactive.
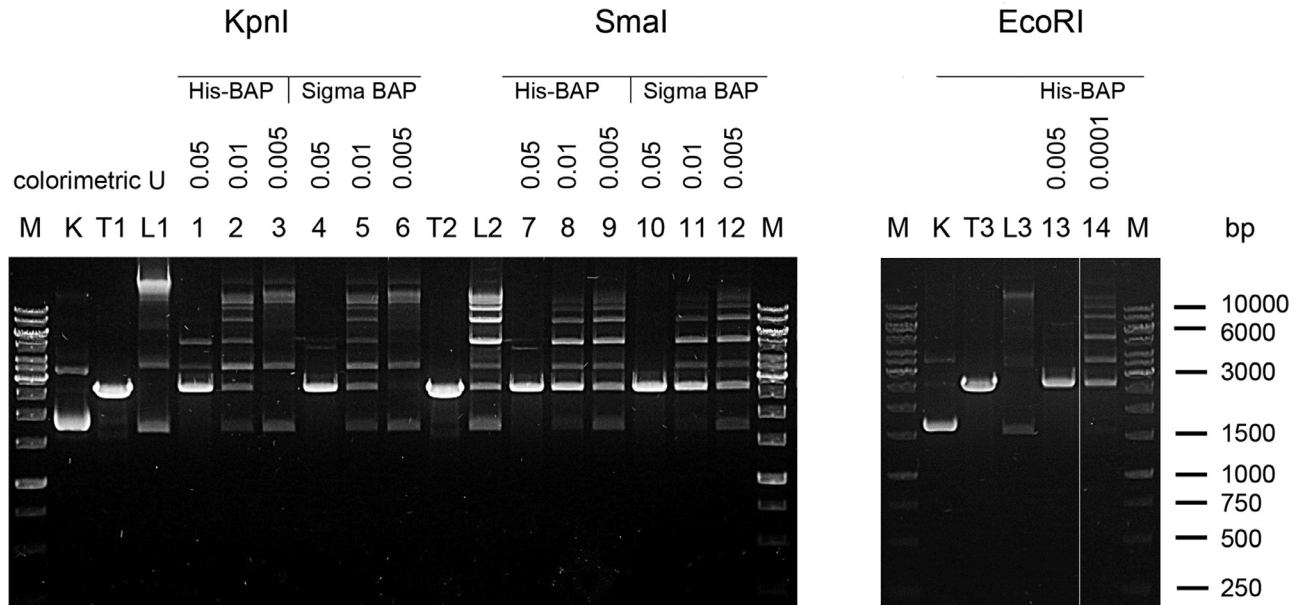
Fig. 4. Various types of DNA termini dephosphorylation using His-BAP and Sigma BAP. Reaction buffer R was composed of 50 mM Tris–HCl pH 8.0 at 55 °C, 1 mM MgCl$_2$, 0.1 mM ZnCl$_2$. Volume of the reaction mixtures was 60 μl. Each reaction mixture contained 1 pmol (app. 1.6 μg) of pUC19 linearized with KpnI, SmaI or EcoRI and His-BAP or Sigma BAP. Linearized pUC19 DNA was purified prior to the dephosphorylation. Following incubation at 55 °C for 45 min, the reactions were stopped and purified by DNA Clean-Up kit and self-ligated in a 30 μl ligation buffer supplemented with 5% PEG4000, using 10 Weiss U of T4 DNA ligase. The reactions were stopped by heating at 70 °C for 5 min and the addition of SDS-containing loading dye/buffer and subjected to 1.0% agarose gel electrophoresis in TAE buffer. Lanes M, GeneRuler 1 kb DNA Ladder; lane K, supercoiled pUC19; lane T1, KpnI-linearized pUC19; lane L1, selfligated KpnI-linearized pUC19 without previous dephosphorylation; lanes 1–3, KpnI-linearized pUC19 dephosphorylated with a serial dilutions of His6-BAP and selfligated; lanes 4–6, KpnI-linearized pUC19 DNA dephosphorylated with a serial dilutions of Sigma BAP and selfligated; lane T2, SmaI-linearized pUC19; lane L2, selfligated SmaI-linearized pUC19 DNA without previous dephosphorylation; lanes 7–9, SmaI-linearized pUC19 DNA dephosphorylated with a serial dilutions of His6-BAP and selfligated; lanes 10–12, SmaI-linearized pUC19 DNA dephosphorylated with a serial dilutions of Sigma BAP and selfligated; lane T3, EcoRI-linearized pUC19; lane L3, selfligated EcoRI-linearized pUC19 without previous dephosphorylation; lane 13, EcoRI-linearized pUC19 incubated with 0.005 colorimetric U of His6-BAP and subjected to selfligation; lane 14, as in lane 13, except that 0.0001 colorimetric U of His6-BAP was used. Original source of the photograps (Krawczun et al. in Fig. 3 [19]). The figure was modified and additional remarks were added to give more details and insights on the dephosphorylation protocol and to show the comparison of the His-BAP and Sigma BAP activity towards various types of DNA ends.

(4) Isolate and purify the protein using a universal protocol consisting of: (*i*) PEI removal of nucleic acids and acidic proteins; (*ii*) ammonium sulfate precipitation; (*iii*) immobilized-metal affinity chromatography.

Note 3: *There can be minor variations among different protein purification procedures, such as: (i) need to increase salt concentration during PEI precipitation to prevent acidic or DNA-interacting proteins to be immobilized in PEI-nucleic acids complexes; (ii) occasionally, the ammonium sulfate concentration will need to be increased and (iii) the ionic strength and concentration of imidazole in adsorption, washing and elution buffers may need individual optimization.*

(5) Reactivate the purified protein to fold into an enzymatically active conformation.

Note 4: *Refolding capability and conditions for various proteins vary substantially and needs to be determined individually. As a general guideline, several factors can help in refolding: (i) physiological* pH*; (ii) selecting optimal ionic strength; (iii) the presence of hydrogen bonding stimulatory compounds, such as* NH_4^+ *ions, (iv) the presence of cofactors; (v) the presence of substrates; (vi) the presence of products; (vii) the presence of non-ionic detergents.*

Protocol for DNA dephosphorylation

(1) Cleave the vector DNA with a restriction endonuclease selected for insertion of the heterologous DNA fragment.

Note 5: *The restriction endonuclease should generate cohesive DNA ends compatible with those present in the insert DNA. Alternatively, either the vector and insert DNA can be cut with blunt-end generating restriction endonucleases, or the 5′- and 3′ DNA termini can be blunted with T4 DNA Polymerase in the presence of high concentrations of dNTPs (usually 0.5 mM).*
Note 6: *In the case of using alternative cloning methods, such as seamless cloning without the use of restriction endonuclease, follow the manufacturer protocol and dephosphorylate vector DNA at the suggested stage of the protocol.*

(2) Stop the reaction and remove restriction endonucleases by one of the following: (i) heat treatment; (ii) phenol/chloroform extraction followed by ethanol precipitation or (iii) a silica-based DNA clean-up kit.

Note 7: *The approach (i) is a good shortcut. Since restriction endonuclease buffers contain* $MgCl_2$ *and have* pH *not far away from His6-BAP dephosphorylation buffer, it is sufficient, when the reaction mixture is diluted 4-fold with 1 x dephosphorylation buffer.*

(3) Dephosphorylate the vector DNA with 5–10 'SmaI cloning units' per 1 mg of approx. 3 kb DNA for 45 min at 55 °C.

Note 8: *If much larger vectors are used, the amount of His6-BAP can be proportionally adjusted, although it is not obligatory, as the His6-BAP is an ultrapure enzyme, thus contains no non-specific nucleases.*

(4) As His6-BAP is a thermostable enzyme, it needs to be carefully removed, to prevent the dephosphorylation of insert DNA. Use either: (i) phenol/chloroform extraction followed by ethanol precipitation or (ii) a silica-based DNA clean-up kit.

Method validation

A validation of the presented method is described by Krawczun et al. [19]. The technology has been validated so far by the successful application to overcome toxicity by engineering the *E. coli* BAP-coding gene *phoA*. This was achieved using the following scheme: (*i*) removal of the DNA coding for a secretion leader; (*ii*) its replacement by a DNA coding for His6-tag; (*iii*) cloning and expression under the strong P_{BAD} promoter; (iv) purification of the engineered enzyme and (*v*) *in vitro* reactivation of BAP activity to the level of that observed in native BAP.

Conclusions

(1) A general concept for the method for the transient inhibition of toxicity of secretory recombinant proteins expressed in *E. coli* was developed.

(2) The method was exemplified by engineering the bacterial alkaline phosphatase *E. coli phoA* gene by removal of the secretion leader and its replacement with a His6-tag, followed by cloning under the strong P_{BAD} promoter control, which resulted in the cytoplasmatic expression of the recombinant His6-BAP.

(3) A very high level of inactive His6-BAP overproduction was obtained as a result of the use of P_{BAD} promoter and transient suppression of His6-BAP toxicity. Our strategy has prevented necessary disulfide bridge formation in the reducing environment of the cytoplasm.

(4) A straightforward 3-step protocol of purification to homogeneity of His6-BAP, suitable for biotechnology production, was established.

(5) An *in vitro* His6-BAP enzymatic activity reactivation protocol was developed, which included: gentle oxidation in redox buffer in the presence of cofactors (Zn^{2+}, Mg^{2+}), reaction product (phosphate) and non-ionic detergents.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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